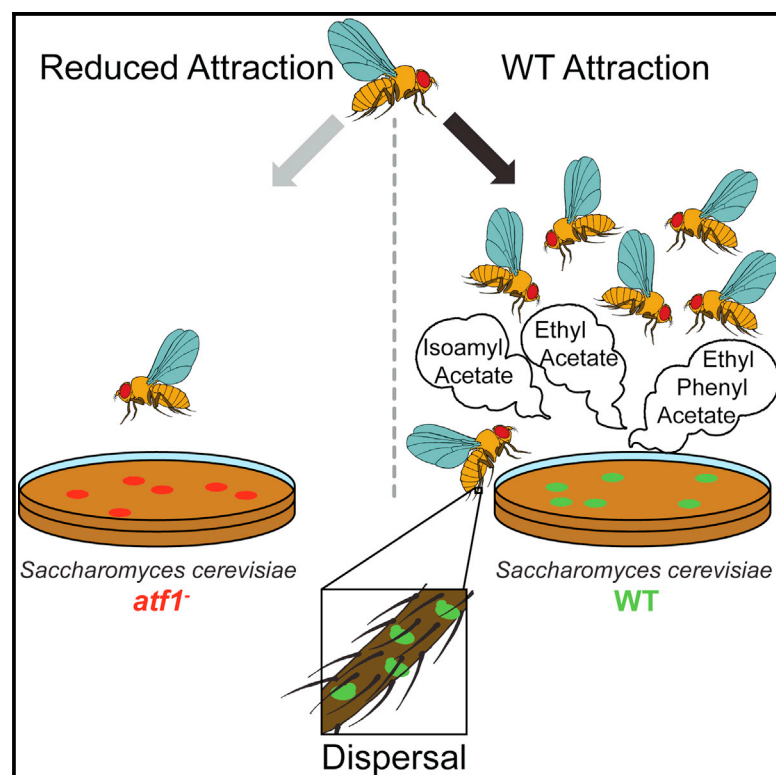


The Fungal Aroma Gene *ATF1* Promotes Dispersal of Yeast Cells through Insect Vectors

Graphical Abstract



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In Brief

Yeast cells produce several different volatile acetate esters. Whereas these fruity aroma compounds are key contributors to the pleasing aroma of fermented beverages like beer and wine, their physiological role for the yeast cells that produce them remains unknown. Christiaens et al. show that two acetate esters, ethyl acetate and isoamyl acetate, help to attract fruit flies that serve as vectors that promote dispersal of the yeast cells. Deletion of the yeast *ATF1* gene, encoding a key acetate ester synthase, drastically reduces *Drosophila* attraction and therefore limits yeast dispersal.

Highlights

The *S. cerevisiae ATF1* gene controls the production of volatile acetate esters

Aroma of *ATF1* mutants elicits different neuronal activity in the fly antennal lobe

Flies are significantly more attracted to wild-type yeast than to *atf1*-null mutants

Addition of isoamyl acetate and ethyl acetate restores attraction of *Drosophila*



The Fungal Aroma Gene *ATF1* Promotes Dispersal of Yeast Cells through Insect Vectors

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SUMMARY

Yeast cells produce various volatile metabolites that are key contributors to the pleasing fruity and flowery aroma of fermented beverages. Several of these fruity metabolites, including isoamyl acetate and ethyl acetate, are produced by a dedicated enzyme, the alcohol acetyl transferase *Atf1*. However, despite much research, the physiological role of acetate ester formation in yeast remains unknown. Using a combination of molecular biology, neurobiology, and behavioral tests, we demonstrate that deletion of *ATF1* alters the olfactory response in the antennal lobe of fruit flies that feed on yeast cells. The flies are much less attracted to the mutant yeast cells, and this in turn results in reduced dispersal of the mutant yeast cells by the flies. Together, our results uncover the molecular details of an intriguing aroma-based communication and mutualism between microbes and their insect vectors. Similar mechanisms may exist in other microbes, including microbes on flowering plants and pathogens.

INTRODUCTION

Microbes produce many secondary metabolites, several of which have strong aromas and are central contributors to the flavor of fermented foods and beverages such as cheese, wine, chocolate, and beer (Styger et al., 2011; Swiegers et al., 2005). Flavor formation has been extensively studied in the common brewer's yeast *S. cerevisiae*, which produces several volatile acetate esters such as ethyl acetate (pear aroma), isoamyl acetate (banana aroma), and phenylethyl acetate (flowery aroma; Lambrechts and Pretorius, 2000; Nordström, 1966; Saerens

et al., 2008; Verstrepen et al., 2003a). These acetate esters are formed in a condensation reaction, catalyzed by an alcohol acetyl transferase (AAT), between acetyl-coenzyme A and various higher alcohols derived from the central carbon and amino acid metabolism. *S. cerevisiae* has two different genes coding for AATases, *ATF1* and *ATF2*, of which *ATF1* controls the bulk of the acetate ester formation (Lilly et al., 2000; Verstrepen et al., 2003b). Although many studies have focused on the optimization of aroma formation, the physiological role of these compounds remains elusive and highly debated (Mason and Dufour, 2000; Saerens et al., 2010). Some reports argue that some esters might help maintain plasma membrane fluidity in low-oxygen conditions where the synthesis of unsaturated fatty acids is impaired (Mason and Dufour, 2000). Other hypotheses suggest that ester synthesis may help tune the redox balance (Malcorps and Dufour, 1992) or that esterification of small organic acids may facilitate their removal from cells through diffusion through the plasma membrane (Nordström, 1964). However, no evidence has been found for any of these hypotheses, and deletion of *ATF1* does not reduce fitness under laboratory conditions (Saerens et al., 2010). Moreover, the most commonly used *S. cerevisiae* laboratory strains show significantly lower production of aroma compounds compared to their wild and industrial relatives (Verstrepen et al., 2003b), suggesting that the synthesis of these compounds has not been selected for under laboratory culture conditions and might rather be related to survival in complex natural environments.

In this study, we set out to investigate acetate ester production in the context of the well-established relationship between yeasts and their insect vectors (Fogleman et al., 1981; Giglioli, 1897; Phaff et al., 1956; Stefanini et al., 2012; Suh et al., 2005). Numerous studies have shown that, unlike fungi that disperse through air (Roper et al., 2010) or motile bacteria, sessile microbes like yeasts depend on animal and especially insect vectors for their dispersal (Francesca et al., 2012; Ganter et al., 1986; Goddard et al., 2010). In turn, microbes serve as a

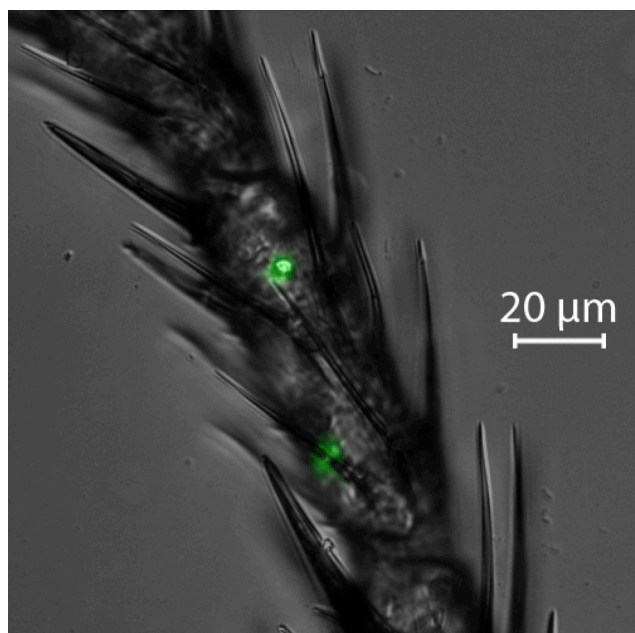


Figure 1. *Drosophila* Transport Yeast Cells in a Laboratory Setup

Yeast cells present on the front leg of a *Drosophila* fly. Sterile individual flies ($n = 11$) were allowed to roam a plate containing a single fluorescent yeast colony (Movie S1) for 1 hr. Afterward, flies were dissected and yeast cells on the various appendages were visualized using fluorescence microscopy. To quantify the number of cells that were transported, each part of the body was washed in a peptone buffer and the buffer was subsequently plated for cell counting (Table S1).

protein-rich food source for these insects and may even boost their immunocompetence (Anagnostou et al., 2010; Gibson and Hunter, 2010; Sang, 1956; Stamps et al., 2012). Previous studies have demonstrated that fermentation can increase the attractiveness of natural substrates toward *Drosophila* (Becher et al., 2012), even though there appears to be a variability in different yeast strains' ability to do so (Palanca et al., 2013). Additionally, multiple insect species are attracted to fruit and plant aroma mixtures that contain acetate esters (Boch et al., 1962; Galizia et al., 1999; Grosjean et al., 2011; Nojima et al., 2003). Finally, a few seminal studies have demonstrated that *Drosophila* antennae possess specific receptors for certain acetate esters (Elmore et al., 2003; Hallem and Carlson, 2006; Vosshall et al., 2000). Given this complex mutualism between microbes and insects, it seems likely that mechanisms evolved to facilitate these interactions. In this study, we show that a specific yeast gene, *ATF1*, produces acetate esters that change the neural representation of yeast odor in the *Drosophila* antennal lobe and help to attract insect vectors.

RESULTS

Yeast Strain Characterization

Experiments to investigate whether acetate esters help to attract insect vectors were carried out using two biological model systems: the fruit fly *Drosophila melanogaster* and the brewer's yeast *Saccharomyces cerevisiae*. To test the suitability of these

organisms for our research, we demonstrated that *Drosophila* do transport yeast cells in a laboratory setup and can indeed act as a vector (Figure 1; Movie S1; Table S1). However, the most commonly used *S. cerevisiae* lab strain, S288c, produces much less aroma compared to wild and industrial *S. cerevisiae* strains (Verstrepen et al., 2003b), making it a poor reference strain for this study. Hence, in order to select a suitable strain, we performed fermentations with 285 genetically diverse *Saccharomyces sensu lato* strains and analyzed their aroma profile (Figure S1). From this collection, we selected a representative diploid strain, coded Y182, for our experimental setup. This *S. cerevisiae* strain was isolated from a vineyard and has an average production of both isoamyl as well as ethyl acetate. Moreover, the strain also shows a relatively high efficiency for genetic transformation, which enabled us to use genetic engineering to create *ATF1* deletion mutants. We deleted both alleles of *ATF1* in this diploid strain and subsequently analyzed the concentration of aroma compounds in the fermentation products of the wild-type and null mutant strains, focusing on higher alcohols, acetate esters, and medium-chain fatty acid esters, as well as ethanol and acetic acid levels, compounds that have been proposed to attract *Drosophila* (Hallem and Carlson, 2006; Hallem et al., 2004). These analyses confirmed that the levels of various acetate esters were severely reduced or even completely abolished in the *atf1*[−] mutant strain, whereas no significant differences in the concentration of other volatile compounds such as acetaldehyde or higher alcohols was observed (Table 1). The effect of the presence of *ATF1* on the yeast's fitness was investigated using an automated multimode plate reader as well as automated time-lapse microscopy. In keeping with previous studies (Saerens et al., 2010), no difference in the growth rate between the wild-type (WT) and *atf1*[−] mutant was observed in liquid or on solid media (see Supplemental Information for more information).

The Presence of *ATF1* Leads to Increased *D. melanogaster* Attraction

To evaluate whether changes in the yeast's aroma profile caused by the deletion of the *ATF1* gene lead *D. melanogaster* to prefer one strain over the other, we used an olfactory behavioral assay in a specially constructed arena (Figure 2). In this computer-controlled system, different airstreams can be released independently from each other out of the four corners of the isolated arena and cleared through a vacuum, applied in the center of the arena (Figure 2A). In this setup, the aromas from two different fermentations are delivered from opposing corners, whereas odorless air is streamed from the two remaining corners. For each olfactory behavioral assay, 50 flies were released in the arena and their positions were recorded with a camera placed above the arena. Subsequently, automated image analysis was used to quantify the number of flies in each of the four quadrants, before, during, and after odor delivery. Based on these numbers, a preference index was calculated for each input (see Figure 2 for details). During the first 2 min of each experiment, odorless air was delivered from all four corners of the arena. During this period, flies did not show preference for any specific quadrant. However, as soon as the airflow was switched so that two out of four corners contained the aroma of WT and *atf1*[−] mutants

Table 1. Aroma Production by WT and *ATF1*[−] Strain

Compound (ppm)	WT-Y182	SD	<i>atf1</i> [−]	SD	p Value
Acetaldehyde	8.98	2.73	9.67	2.89	0.74
Ethyl acetate	14.54	1.53	6.45	0.43	5.20 × 10^{−5a}
Ethyl propionate	0.08	0.01	0.09	0.01	0.19
Ethyl isobutyrate	0.02	0.00	0.02	0.00	0.90
Propyl acetate	0.03	0.01	ND	ND	a
Isobutyl acetate	0.09	0.03	ND	ND	a
Ethyl butyrate	20.24	3.26	20.40	2.23	0.94
Isobutanol	13.38	2.57	13.31	1.60	0.96
Isoamyl acetate	0.82	0.20	0.13	0.06	5.68 × 10^{−4a}
Butanol	55.45	2.21	57.67	5.22	0.53
Isoamyl alcohol	37.71	5.69	41.54	3.92	0.31
Ethyl hexanoate	0.13	0.03	0.18	0.01	0.04
Ethyl octanoate	1.08	0.23	1.14	0.13	0.66
Phenyl ethyl acetate	0.10	0.02	ND	ND	a
Phenyl ethanol	0.13	0.06	0.08	0.03	0.16
Ethanol (% v/v)	6.50	0.05	6.46	0.02	0.14
Acetate (g/l)	24.75	6.25	22.15	3.69	0.32

Concentrations of aroma compounds (in parts per million) produced by the diploid WT Y182 strain and the isogenic *ATF1* double-deletion mutant during fermentation (see Figure S1 for more information on Y182). For each strain, four fermentations were performed and each was analyzed in four technical replicates. Acetate esters are displayed in bold. These results demonstrate that the only compounds significantly affected by the *ATF1* deletion are acetate esters (ND, not detected). Production of these aroma compounds by non-*Saccharomyces* strains may be found in Table S2.

^aSignificant difference between the WT and deletion strain ($p < 0.05$).

(each in one opposing corner), the flies significantly preferred the quadrant with the WT aroma over the aroma of *atf1*[−] mutants (p value $< 1 \times 10^{-4}$). Afterward, when the airstream at all corners was switched back to odorless control, the flies no longer preferred a specific quadrant and were once again distributed randomly (Figure 2; Movies S2 and S3).

These results indicate that *Atf1* activity has a significant effect on the attraction of *Drosophila* by the yeast cells. To verify whether the observed behavioral differences are indeed due to the lower acetate ester levels produced by the *atf1*[−] mutant, we supplemented the *atf1*[−] fermentation medium with phenylethyl acetate, isoamyl acetate, and ethyl acetate (the three important acetate esters most affected by the *ATF1* deletion), so that the final concentration of each ester was equal to that in the WT fermentation medium. We then repeated the olfactory preference tests with this supplemented medium pitted against the fruity WT medium, and no significant difference in preference for either medium was observed, clearly illustrating the central role of phenylethyl, isoamyl, and ethyl acetate in the altered attraction phenotype (Figures 2C and 2D). To further tease apart the specific contribution of each ester, we supplemented the *atf1*[−] medium with each ester separately and tested these against the WT medium. These experiments revealed that ethyl acetate is the most important compound, because addition of this ester is sufficient to

make the *atf1*[−] medium as attractive as the WT medium in our olfactory behavioral assay. Addition of isoamyl acetate resulted in increased attraction compared to the unsupplemented *atf1*[−] medium, but the WT attraction was not fully restored. Finally, phenylethyl acetate did not significantly affect *Drosophila* behavior.

To further confirm the role of the yeast *ATF1* gene in *Drosophila* attraction, we set up a second series of preference experiments, this time in larger cages (30 × 30 × 20 cm), allowing flies more freedom of movement in three dimensions, which has been implicated to alter their behavior compared to setups where their movement is more restricted (Becher et al., 2010; Lebreton et al., 2012). Analogous with the olfactory assay in the smaller arena, we placed traps containing either WT or *atf1*[−] mutant fermentation medium in opposing corners of the larger cages and two traps containing only water in the two remaining corners. To account for position effects, the traps were switched across different repeats. Our results further confirmed a clear preference of *D. melanogaster* for the WT strain, attracting 2-fold more flies than the *atf1*[−] strain (see Figure S2 for details).

WT and *atf1*[−] Aromas Are Perceived Differently by *Drosophila*

To further evaluate the effect of isoamyl and ethyl acetate on the *Saccharomyces-Drosophila* interaction, we used in vivo calcium imaging to measure the neuronal responses to *atf1*[−] and WT yeast aroma in the antennal lobe of a *Gal4-GH146/UAS-GCaMP6m*; *Drosophila* strain expressing the transgenic calcium indicator GCaMP6m in its projection neurons (Figures 3A and S3A; Chen et al., 2013). These measurements show a clear alteration in the response of projection neurons, which receive direct olfactory input from olfactory sensory neurons, to the mutant yeast strain compared to the WT strain (Figure 3B). To quantify the differences in the olfactory representation of the different yeast strains, we compared the odor-induced neuronal responses using pairwise Pearson's correlations (Figure 3C). Moreover, we further analyzed these activity maps by comparing the autocorrelation of the repetition of the WT yeast odor across different trials to the cross correlation of the odor representations of WT yeast against *atf1*[−] yeast and *atf1*[−] yeast supplemented with the missing esters (Figure S3E). Both analyses revealed that the WT and *atf1*[−] yeast odor are represented by significantly different activity patterns in the fly antennal lobe. Moreover, we observed that the addition of each ester in physiological concentrations to the *atf1*[−] mutant yeast medium shifts the evoked antennal lobe activity more toward the WT activity pattern, as evident by the significant increase in correlations between WT and *atf1*[−] yeast odors when esters are supplemented. Furthermore, the principal component analysis of the antennal lobe odor responses shows that supplementing *atf1*[−] with esters shifts the representation of *atf1*[−] yeast odor toward WT yeast odor (Figure 3F). In summary, all these analyses confirms that *atf1*[−] yeast odor and the WT yeast odor are represented differently in the *D. melanogaster* antennal lobe and the odor representations of *atf1*[−] yeast become more similar to WT yeast when each ester is supplemented to the same levels as found in the WT medium.

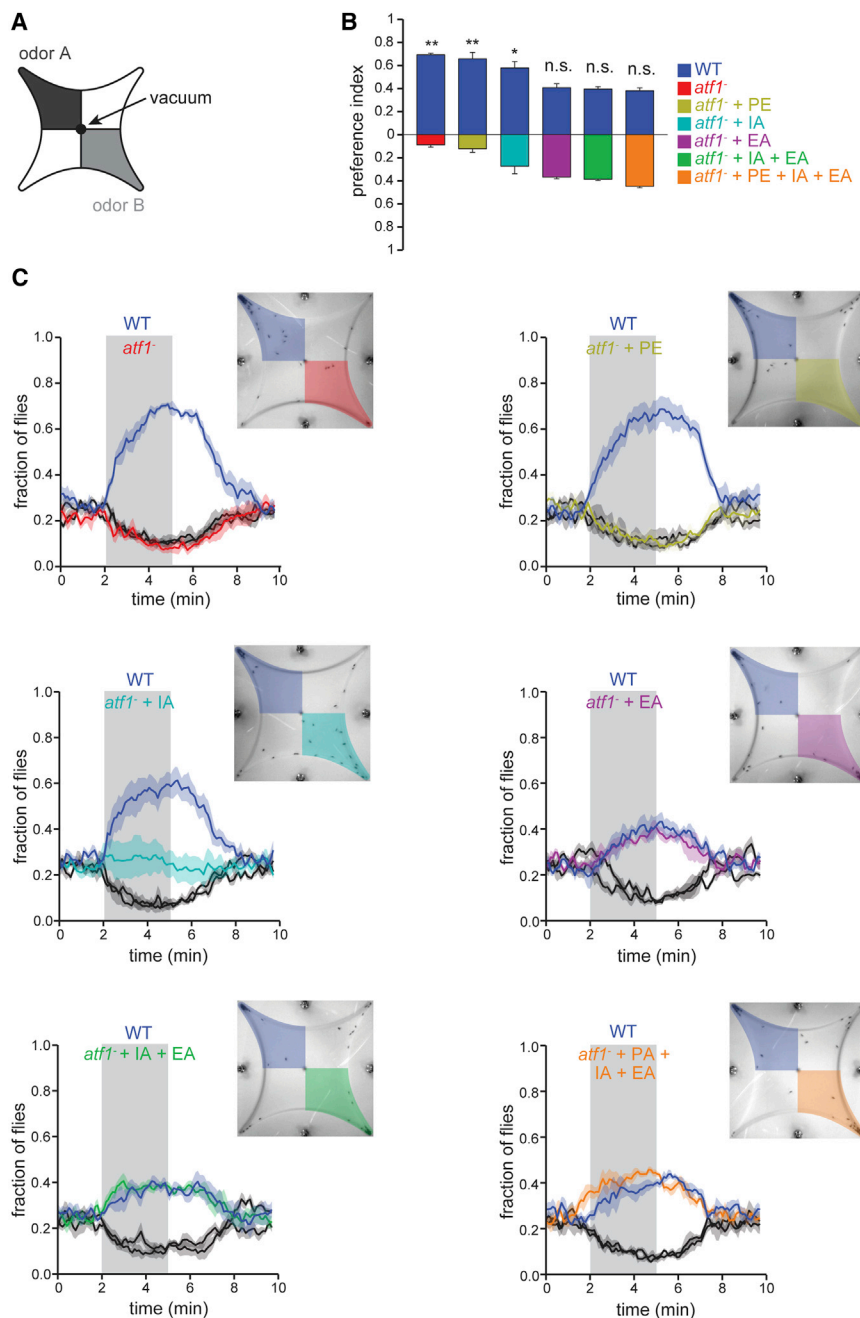


Figure 2. Volatile Acetate Ester Production Affects the Attractiveness of Yeast Cells for *Drosophila melanogaster*

(A) Experimental arena populated with fifty 20 hr-starved *D. melanogaster* CS10 flies. Air is flown from the four corners of the arena, with a common outlet in the center, generating four quadrants that each have their own air profile. Fly movements were recorded during 10 min experiments (odorless air for 2 min from each of the four corners, odors for 3 min from two opposing corners, and odorless air from the other two corners, and finally 5 min of odorless air from all four corners; see also main text for details and [Movies S2](#) and [S3](#)).

(B) Preference index defined by the fraction of flies being present in the corresponding odorized quadrant during the last minute of odor exposure. The odors of WT yeast and *atf1*⁻ yeast supplemented with ethyl acetate (EA) and isoamyl acetate (IA) (at concentrations that match the levels present in the WT yeast) exhibit a higher preference index than *atf1*⁻ yeast odor whereas *atf1*⁻ supplemented with phenylethyl acetate (PA) does not (paired t test; *p < 0.02; **p < 0.001). No significant difference was found when WT yeast odor was competed against *atf1*⁻ yeast odor supplemented with all three esters or with ethyl acetate and isoamyl acetate or with ethyl acetate. The error bars represent SEM. n.s., not significant.

(C) Temporal course of number of flies per quadrant for the different experimental conditions. Colors correspond to the respective media (air shown in black/gray). Shaded traces represent SEM for the number of flies (n = 200 flies; four experimental sessions; three trials/odor/session). The gray rectangle indicates the time of odor exposure. Inset figures correspond to snapshots taken at the end of the odor delivery period (5th min). For the large cage setup, see [Figure S2](#).

Expression of *ATF1* Results in Increased Yeast Dispersal

The previous experiments revealed that the *ATF1*-mediated production of acetate esters has a significant impact on the attractiveness of yeast cells toward *D. melanogaster*. However, this does not necessarily imply a benefit for the yeast, because increased attraction of flies might merely result in more yeast cells being consumed by the flies. However, we hypothesized that increased fly attraction might also result in increased dispersal of the yeast cells through their insect vectors. To study whether expression of *ATF1* affects yeast dispersal by fruit flies, both the WT and *atf1*⁻ mutant strain were fluorescently labeled

by overexpressing either yECitrine or mCherry. These labeled strains were inoculated on one of two designated spots on a specially constructed YPD plate, equidistant from the center of the plate ([Figure S4](#)). For each strain, the inoculum contained 1×10^6 cells, corresponding to the amount of *Saccharomyces* cells that may be found on infected grapes ([Mortimer and Polsinelli, 1999](#)). Subsequently, a fly was allowed to roam around the test environment overnight in complete darkness, after which it was removed.

Then, the spots containing the initial inocula were removed as well so that only dispersed yeast cells remained and the plates were incubated to allow growth of these cells. After 48 hr, the plates were washed and the relative presence of each strain was quantified using a flow cytometer. Because there is no growth difference between the WT and mutant strains ([Supplemental Information](#)), the relative presence of each strain indicates the ratio in which the two strains were transported by the fly. Analysis of 100 independent experiments revealed that the fruity WT

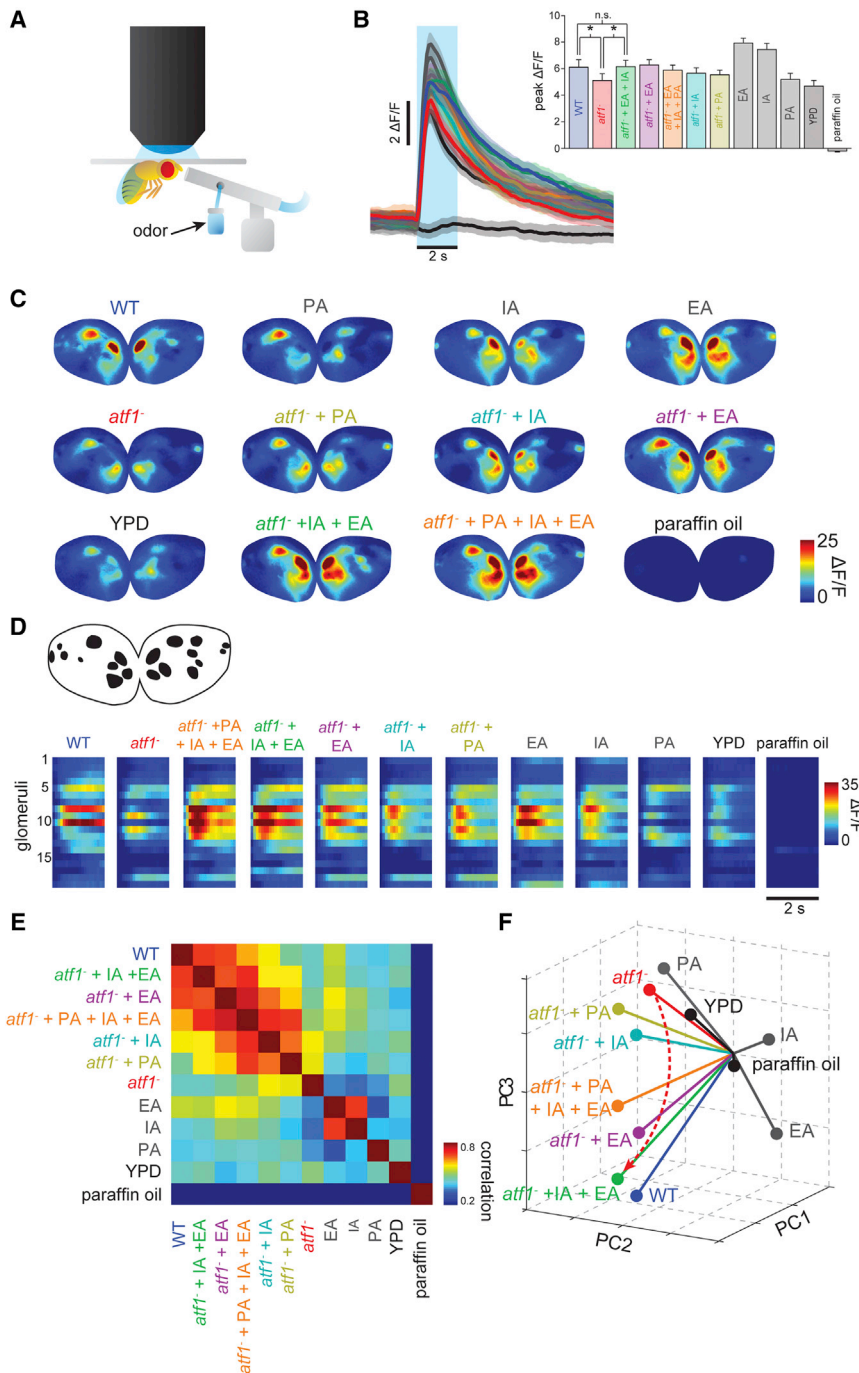


Figure 3. WT and *atf1*⁻ Yeast Odors Are Represented Differently in the Antennal Lobe of *Drosophila melanogaster*

(A) Experimental setup depicting a fly expressing the transgenic calcium indicator GCaMP6 in projection neurons. Briefly, the fly is fixed to an aluminum chamber with wax and the cuticle above the antennal lobes is removed, which allows imaging of the antennal lobes from the dorsal side. A tube is placed in front of its antennae for odors to be delivered (carrier stream flow rate = 2,000 ml/min).

(B) Temporal course of the average fluorescence signal in response to different odors in the entire antennal lobe. Shadowed traces represent SEM for the change in fluorescence. The blue rectangle depicts the 2 s time window after response onset, used for quantitative analyses. Inset bar plot shows that pure *atf1*⁻ yeast odor and *atf1*⁻ odor supplemented with PA elicit significantly weaker responses than WT yeast odor and *atf1*⁻ yeast odor supplemented with EA and IA, respectively (n = 14 flies; *p < 0.02; paired t test).

(C) Representative antennal lobe activity maps corresponding to different yeast and ester odors as well as paraffin oil as control, respectively. Calcium responses were averaged during the 2 s window shown in (B).

(D) Regions of interest manually assigned to each responsive glomerulus based on activity maps for the fly shown in (C). On the right, the corresponding glomerulus-based response profiles for the different odors and control are shown. Profiles were constructed for the 2 s window shown in (B).

(E) Correlation matrix representing the pairwise similarities for the different odor response profiles (n = 14 flies). Note that *atf1*⁻ yeast odors supplemented with esters are more correlated to WT yeast odor than *atf1*⁻ yeast odor alone.

(F) Principal component analysis of the odor response profiles. The first three components are shown (total variances explained by first three principle components [PCs] = 79.6%; PC1 = 59.4%; PC2 = 12.8%; PC3 = 7.4%). Upon addition of more esters, the *atf1*⁻ yeast odor representations are progressively pushed in the direction of the representations of the WT yeast odor, indicating a more-similar response. Additional analyses are in Figure S3.

strain was on average transported four times more than the *atf1*⁻ mutant (Figure 4). A separate mirror experiment (n = 100) demonstrated that the nature of the fluorescent tag did not influence the dispersal of either strain (Supplemental Information).

DISCUSSION

Together, these results demonstrate that expression of the fungal alcohol acetyl transferase gene *ATF1* promotes attraction

of *D. melanogaster*, which in turn promotes dispersal of the yeast cells by the insect vector. Using a combination of molecular, behavioral, and neurobiological tests, we show that acetate esters produced by yeasts change the antennal lobe odor representations of *Drosophila melanogaster* and greatly increase its attraction to yeast. Moreover, we identify two acetate esters, ethyl acetate and isoamyl acetate, as the main signaling molecules associated with this phenotype.

It is interesting to note that compounds like ethyl acetate and isoamyl acetate render ripening fruits their typical aroma (Vermeir et al., 2009). It is therefore tempting to speculate that yeasts

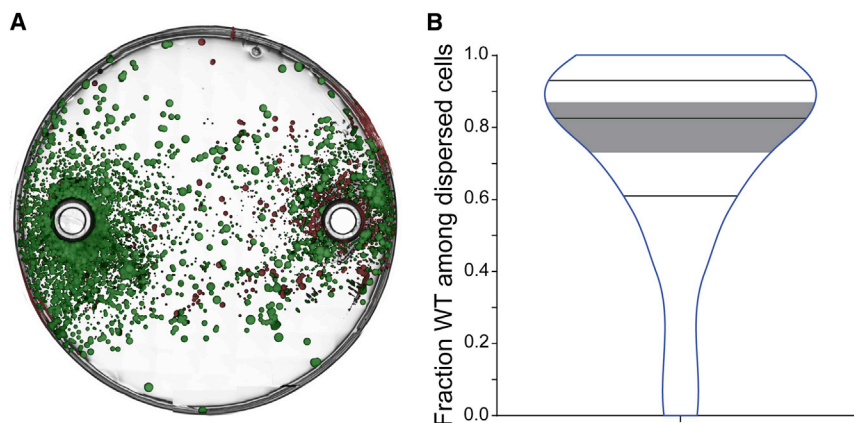


Figure 4. Fruity Yeast Strains Get Dispersed More by *D. melanogaster*

Two fluorescently labeled strains (green or red) were plated equidistantly from the center of a YPD agar plate (black circles; see also Figure S4 for details). A 20 hr-starved CS10 fly was introduced to the plate, which was closed and placed in a container, shielded from external light. After 14 hr, the fly was sedated and removed from the plate, after which the plate was incubated for 48 hr at 30°C to allow growth of dispersed yeast cells. Then, the spots from the initial inoculum were removed and the remaining cells were washed off the plate and analyzed using a flow cytometer.

(A) Image of a representative plate after 48 hr of incubation, before washing. The WT strain is yECitrine-labeled (green-yellow) and the *atf1*[−]

strain mCherry-labeled (red). The image clearly shows that the *atf1*[−] (red) strain shows reduced dispersal by the flies.

(B) Violin plot of the fraction of dispersed WT cells ($n = 95$, excluding five samples where no cells were dispersed); black lines indicate quartiles and median. Fruity WT cells were dispersed significantly more than *atf1*[−] cells, shown by the fact that the estimated fraction (gray band = 95% confidence interval) is significantly higher than the 50% expected if dispersal was equal for both (binomial mixed model; p value $< 1 \times 10^{-10}$; details in Experimental Procedures).

have evolved the capacity to synthesize these esters to mimic this aroma in order to attract flies. However, it is difficult to show that the yeast *ATF1* gene specifically evolved to stimulate the production of aroma compounds with the aim of attracting insects. Nonetheless, there are compelling arguments for this hypothesis, because several reports demonstrate the intricate mutualism between yeasts and flying insects like fruit flies (Ganter et al., 1986; Giglioli, 1897; Gilliam et al., 1974; Phaff et al., 1956; Reuter et al., 2007; Starmer et al., 1986; Stefanini et al., 2012). For the yeast, dispersal is essential to reach new niches, especially when nutrient levels are running low. Under these conditions, the center of yeast colonies is expected to be a hypoxic environment (Cáp et al., 2012) and hypoxic conditions may increase *ATF1* expression (Fujii et al., 1997), which in turn results in increased acetate ester formation (Lilly et al., 2000; Malcorps and Dufour, 1992; Verstrepen et al., 2003b). Hence, the production of aroma compounds like isoamyl acetate could help alert flies to the presence of yeast cells, a vital component of their diet. Whereas some of the yeast cells are consumed by the insects, a fraction of cells will stick to the fly body and get dispersed to a different environment. Furthermore, yeasts might reap more benefits from this interaction than mere dispersal. Low nutrient levels trigger the formation of spores that can survive passage through the fly gut and promote outbreeding and thus genetic variability (Freese et al., 1982; Reuter et al., 2007). Additionally, it has been demonstrated that certain insects, such as wasps, act as a reservoir for yeasts when environmental conditions are harsh, for example, during a cold winter (Stefanini et al., 2012).

A last important question is whether aroma-based attraction of insects is limited to *S. cerevisiae*. In a first preliminary assay, we observed that many different yeasts can be isolated from the body of *Drosophila* isolated from natural environments and that the vast majority of these yeasts produced aroma-active esters (Table S2). Moreover, we also isolated several strong aroma-producing yeasts from flowers. These preliminary results suggest that aroma production is not restricted to *S. cerevisiae* and may be a much more general theme in microbe-insect inter-

actions. This is further supported by other studies that reported the isolation of many different yeast species from various insects and the discovery that receptors for acetate esters are widespread in insects (Galizia et al., 1999; Phaff et al., 1956). The formation of fungal aroma compounds may even play a role in mimicry and the relation between plants and their pollinators (Dentinger and Roy, 2010; Stökl et al., 2010).

Lastly, our study also highlights the limitations of using standard laboratory conditions with pure single-species cultures. Clearly, studying model organisms in a more complex ecological context will increase our understanding of physiology and may help to reduce the number of genes with unknown functions.

EXPERIMENTAL PROCEDURES

Strains

Both *ATF1* alleles in the Y182 strain were deleted using deletion cassettes based on pUG6, conferring resistance to either Hygromycin B or G-418 disulfate. Both markers were removed through the Cre/LoxP technique using pSH65. Deletions as well as marker removal were confirmed through (lack of) growth on selective media, as well as PCR (primers in Table S3). Fermentations performed with WT and mutant strains were analyzed using a head-space-gas chromatography-flame ionization detector system; ethanol and acetate levels were determined using enzymatic kits. Growth rates in liquid YPD were measured in an automated multimode plate reader, whereas growth rates on solid media were determined using automated time-lapse microscopy (New et al., 2014). Differences between strains were analyzed using Mann-Whitney U tests.

Olfactory Preference Assays

Prior to testing, 50 CS10 flies were starved for 20 hr at ~25°C in scintillation vials containing a wet filter paper and then placed in the four-input arena. A camera placed above the arena filmed each trial, each lasting 10 min (2 min, odorless air; 3 min, odor exposure; 5 min, odorless air); each set of 50 flies was tested three times. The number of flies in each quadrant was detected by an automated MATLAB algorithm, based on binarized images. Paired t tests were used for statistical comparisons. For the assays in large cages, 20 CS10 flies were starved for 20 hr at 25°C in vials containing 2% agarose. Tests lasted 5 hr, after which all flies were sedated and the number of flies in each trap was counted. Statistical analyses were performed using a generalized linear mixed model (GLMM), available in R-package lme4. For the calcium

imaging experiments (Yaksi and Wilson, 2010), principle-component analyses and Pearson's correlations were calculated on the antennal lobe images obtained during 2 s after response onset windows. For statistical analyses, paired t tests were performed on antennal lobe peak responses and Mann-Whitney for correlation coefficients comparisons.

Dispersal Experiments

Flies were starved for 20 hr at 25°C in vials containing 2% agarose. Dispersal experiments were performed with custom-made plates (see Figure S4) filled with YPD-2% agar. Statistical analyses of the dispersal of both strains were performed using a GLMM, available in R-package lme4. Significance of deviation from 50:50 was tested using a Wald z test, also available in lme4.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, three tables, and three movies and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.09.009>.

AUTHOR CONTRIBUTIONS

J.F.C. and L.M.F. designed, performed, and analyzed experiments and wrote the manuscript. T.L.C. performed experiments. L.D.M., J.M., T.W., and B.A.H. helped with design and interpretation of results and wrote the manuscript. K.J.V. conceived the study, and E.Y. and K.J.V. designed and interpreted experiments and wrote the manuscript.

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